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Carbonyl reductase of *Candida parapsilosis* – Stability analysis and stabilization strategy

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ABSTRACT

The homodimeric *Candida parapsilosis* carbonyl reductase 2 (CPCR2) is an industrially attractive biocatalyst due to its broad substrate range and high stereoselectivity. In addition, CPCR2 is reasonably stable in monophasic organic solvents (e.g. alcohols) but apparently instable in biphasic organic systems. Hence, we conducted first a thorough quantitative inactivation study of CPCR2, using both wild-type and stability improved variants, in an attempt to identify critical factors influencing the enzyme stability. Possible inactivation phenomena including oxidation, shear forces, dissociation and adsorption at interfaces were assessed on a microliter scale using quantitative kinetic assays. Our results demonstrate that interface interactions and dimer dissociation are the main reasons for inactivation of CPCR2. Shear forces seems to enhance these inactivation processes whereas oxidation plays no role in CPCR2 inactivation. Secondly, an attempt was made to find suitable stabilization strategies to utilize CPCR2 in various reaction systems. To minimize the inactivation, bovine serum albumin was used as traditional blocking and crowding agent. The residual activity of the wild-type was successfully increased up to 2.5-fold by addition of 1 $\mu g\,mL^{-1}$ bovine serum albumin. To avoid dimer dissociation the cofactor concentration was successively increased. The residual activity was successfully enhanced up to 5-fold, 3-fold and 1.5-fold for the wild-type, single and double mutant, respectively. Further, recently gained data from the enzyme crystal structure were used to interpret the effects of stabilization. We propose conformational change of a flexible region in CPCR2 upon binding of the cofactor leading to internal stabilization of the enzyme. In conclusion, we propose the addition of bovine serum albumin and the cofactor NADH as a suitable stabilization strategy to utilize CPCR2 in various reaction systems.

1. Introduction

The asymmetric reduction of carbonyl compounds by enzymatic catalysis is of particular interest in the synthesis of pharmaceuticals [1–3]. However, only few alcohol dehydrogenases or carbonyl reductases are known so far to convert sterically demanding substrates into valuable hydroxy ketones [4]. One notable example therefore is the homodimeric *Candida parapsilosis* carbonyl reductase 2 (CPCR2). This reductase displays the ability to convert an exceptionally broad substrate range and shows high stereoselectivity [5,6]. CPCR is already known for more than 20 years and was first identified by Peters and coworkers [7,8]. At that time a mixture of at least two enzymes was purified, as recently suggested by Jakoblinnert et al. [9]. They investigated different CPCR preparations comparing their biochemical features and substrate spectra to discriminate between different isoenzymes. Results showed that CPCR preparations may be rather classified into a mixture of CPCR1 and CPCR2 having a narrow and a broad substrate range, respectively. They verified CPCR2 to be the enzyme of interest and identical with a secondary alcohol dehydrogenase from *C. parapsilosis* (CpSADH) identified and published by Kojima and coworkers [10,11].

Abbreviations: ABEE, ethyl 4-acetylbutyrate; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; LB, Protein LoBind Eppendorf tubes; PP, polypropylene; PP-BSA, bovine serum albumin pretreated polypropylene; SG, silanized glass; TEA, triethanolamine; UG, untreated glass.

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CPCR2 is a medium-chain zinc-dependent carbonyl reductase preferring NADH as a cofactor. Man and coworkers recently resolved the crystal structure emphasizing its predominant appearance as homodimeric enzyme with a molecular weight of 36 kDa each monomer [12]. They further investigated the relevance of the two zinc ions bound in each monomer with respect to the enzymes activity and stability. They showed that a relatively harsh treatment with 10 mmol L^{-1} EDTA for 2 h at 4 °C removed the catalytic zinc ions, whereas the structural zinc ions were even stronger bound [12].

The applicability of CPCR2 in highly stereoselective biocatalytic reactions on lab scale is highlighted by an increasing number of publications. Various enzymatic pathways for the production of fine chemicals using CPCR2 working in both aqueous and non-conventional media as neat substrates have been published [13–16]. Most recently CPCR2 was successfully used in a chemo-bio-bio reaction sequence for the production of (S)-γ-valerolactone [17]. Furthermore, CPCR2 has been already applied in different reaction set-ups as in a novel emulsion reactor [18], enzyme-membrane reactors [19,20] and also in whole cell biocatalysis [16,21]. Thereby, CPCR2 was identified to be reasonably stable in monophasic organic solvents (e.g. alcohols), but apparently instable in biphasic organic systems [22] and microemulsions [23]. Different stabilization approaches like rational protein engineering [24], addition of protectants as dithithreitol (DTT) [25] and immobilization [26] were undertaken in literature to enhance stability and activity of CPCR2 under reaction conditions. However, only few statements were given to identify and explain underlying inactivation to achieve target-oriented stabilization of CPCR2 for successful application in lab- and industrial scale.

The first part of this study focuses on a thorough quantitative inactivation study. Based on earlier published suggestions on CPCR2 inactivation mechanisms, possible phenomena including oxidation, shear forces, dissociation or adsorption at interfaces on a microliter scale were assessed using kinetic assays to investigate individual inactivation phenomena on CPCR2 (Fig. 1). Besides the CPCR2 wild-type, two genetically modified CPCR2 variants which are enhanced toward activity and thermal stability [24] were used to distinguish between different inactivation effects. In the second part of our research the attempt was made to find a suitable stabilization strategy verifying the effect of stabilizing CPCR2 by different analytical approaches. The recently solved enzyme crystal structure [12] was further used to interpret the inactivation phenomena on the enzyme. In conclusion, we propose a suitable stabilization strategy to utilize CPCR2 in various reaction systems.

2. Experimental

2.1. Chemicals and organisms

All chemicals were purchased from Sigma–Aldrich GmbH (Steinheim, Germany), Merck KGaA (Darmstadt, Germany), Carl Roth GmbH (Karlsruhe, Germany), Alfa Aesar GmbH (Karlsruhe, Germany) and Life Technologies™ (Darmstadt, Germany) if not stated otherwise. The cofactor β-NADH was purchased from Biomol GmbH (Hamburg, Germany) as reduced sodium salt. Enzyme purification chromatography cartridge and solutions were purchased from IBA GmbH (Göttingen, Germany).

Escherichia coli BL21 (DE3) strains containing strep-tagged gene constructs of CPCR2 wild-type, CPCR2-(A275T) and CPCR2-(A275S, L276Q) variants, respectively, within a pET22b+ vector were used for all experiments ([9,28]; ampicillin resistant). CPCR2 variants were kindly created and provided by the Chair of Biotechnology (Prof. U. Schwaneberg), RWTH Aachen University (Aachen, Germany).

2.2. Enzyme expression and purification

All CPCR2 protein expressions were carried out at pH 7.0 using terrific broth (TB) medium (12 g L^{-1} tryptone, 24 g L^{-1} yeast extract, 5 g L^{-1} glycerol, 12.54 g L^{-1} dipotassium phosphate (K_2HPO_4), 2.31 g L^{-1} monopotassium phosphate (KH_2PO_4)) containing 0.1 g L^{-1} ampicillin. For pre-cultures, 10 mL of TB medium were inoculated using cryo-cultures (25% v/v glycerol). Incubation took place in 250 mL shake flasks at a shaking frequency of 350 rpm, a shaking diameter of 50 mm and 37 °C. Growth was monitored online using the RAMOS technology [27,28].

Pre-cultures were harvested during the exponential growth phase at an oxygen transfer rate (OTR) of about 50 mmol L^{-1} h^{-1} and used for inoculation of the main culture to an initial optical density (OD_{600}) of 0.2. Fermentation was carried out in a BIOSTAT® B plus fermenter (Sartorius Stedim Biotech, Göttingen, Germany) at 37 °C. The dissolved oxygen tension (DOT) was maintained above 30% via varying the agitator speed while keeping the aeration rate constant at 1 vvm. At an OD_{600} of 1.1 protein expression was induced with 1 mmol L^{-1} isopropyl β-D-1-thiogalactopyranoside (IPTG) and the temperature was set to 16 °C for the duration of the production phase (16 h). Cells were harvested through centrifugation (4 °C, 4000 rpm, 20 min) using a Rotina 35 R laboratory centrifuge (Hettich, Tuttlingen, Germany) and pellets were stored at −20 °C. For cell lysis, 5 mL protein extraction reagent Bug Buster®, 1 mg lysozyme and 0.5 mg desoxyribonuclease 1 (DNAse1) were added to 1 g fresh cell weight, mixed thoroughly and incubated for 20 min on ice. The supernatant containing CPCR2 protein was clarified by centrifugation (4 °C, 4000 rpm, 30 min) and subsequently applied to an affinity chromatography using a 5 mL Strep-Tactin Superflow high capacity cartridge H-PR within an Äkta FPLC device (GE Healthcare, Freiburg, Germany). Purification was performed as instructed by the manufacturer using tris(hydroxymethyl)-aminomethane hydrochloride (Tris·HCl, 100 mmol L^{-1}) at pH 8.0 as running buffer. Elution fractions were evaluated for CPCR2 activity, as described in the following paragraph. Fractions showing high enzyme activity were pooled and analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) from Life Technologies™ (NuPAGE® Novex® 4–12% Bis-Tris Protein Gel) for purity. Protein concentration was determined by Bradford and Bicinchoninic acid (BCA) protein assay using bovine serum albumin (BSA) as standard in both assays. To reduce the concentration of interfering substances within the assays 200 μL enzyme stock were diluted 1:20 with deionized water, transferred to an ultrafiltration unit (Sartorius, Vivaspin 6 10000 MWCO PES) and purified by centrifugation (20 °C, 3500 rpm, 30 min) before measurement. The enzyme concentration was always adjusted to 0.7 mg mL^{-1} by addition of glycerol (50% v/v) and stored as enzyme stock solution at −20 °C (henceforth referred to as enzyme stock).

2.3. Activity measurement

All kinetic measurements were performed by using ethyl 4-acetylbutyrate (ABEE) and NADH in a triethanolamine hydrochloride buffer system (TEA, 100 mmol L^{-1}, pH 7.5) at 30 °C. The decrease of the NADH signal was monitored spectrophotometrically at 340 nm (Synergy Mx, BioTek Instruments GmbH, Bad Friedrichshall, Germany) in microtiter plates (96-well MTP, polystyrene, F-profile, Carl Roth GmbH).

To assure reproducible experiments both the MTP and the substrate solution (0.76 mmol L^{-1} NADH; 34.6 mmol L^{-1} ABEE) were preheated to 30 °C for 30 min. The enzyme stock was diluted with TEA buffer accordingly to obtain a linear decrease of absorption. 10 μL of diluted CPCR2 solution were mixed with 190 μL substrate solution using a multichannel pipette assuring synchronized start